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Effects of *vlsE* Complementation on the Infectivity of *Borrelia burgdorferi* Lacking the Linear Plasmid lp28-1

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The loss of linear plasmid lp28-1, which contains the *vls* antigenic variation locus, is associated with reduced infectivity of *Borrelia burgdorferi* in immunocompetent mice. The recombinant shuttle vector pBBE22, which includes the virulence determinant BBE22 from lp25 and restores infectivity to readily transformable *B. burgdorferi* lacking lp25 and lp56, was used to determine the effect of *trans* expression of *vlsE* on virulence. Spirochetes lacking lp28-1 were complemented with the plasmid pBBE22:vlsE, containing both BBE22 and *vlsE*. VlsE protein produced by this construct was expressed and surface accessible in in vitro-cultured *B. burgdorferi*, as determined by surface proteolysis and immunoblot analysis. Clones lacking lp25 but containing lp28-1 and either pBBE22 or pBBE22:vlsE were reisolated consistently from immunocompetent mice 8 weeks after infection. In contrast, a clone lacking both lp25 and lp28-1 and complemented with pBBE22:vlsE was isolated from only a single tissue of one of six C3H/HeN mice 8 weeks postinfection. These results indicate that either an intact *vls* antigenic variation locus or another determinant on lp28-1 is required to restore complete infectivity. In addition, an isogenic clone that retained lp28-1 was complemented with the *vlsE* shuttle plasmid and was examined for *vlsE* sequence variation and infectivity. Sequence variation was not observed for the shuttle plasmid, indicating that the *cis* arrangement of *vlsE* and the *vls* silent cassettes in lp28-1 facilitate *vlsE* gene conversion. Lack of *vlsE* sequence variation on the shuttle plasmid thus did not result in clearance of the *trans*-complemented strain in immunocompetent mice under the conditions tested.

Lyme borreliosis, the most prevalent vector-borne disease in the United States, is a chronic infection caused by *Borrelia burgdorferi* and other members of the genus *Borrelia* (6). Spirochetes are transmitted to mammalian hosts by *Ixodes* ticks, leading to the development of an annular rash called erythema migrans at the site of inoculation and progressing to a multisystemic infection with neurological, arthritic, and cardiac manifestations (45). As infection advances and *Borrelia* disseminate into deeper tissues in the host, a strong immune response is elicited towards the pathogen, including the development of *Borrelia*-specific antibodies (7, 8, 11, 14, 17). Though an active immune response develops early during infection, *Borrelia* is able to escape clearance and persist for months to years. Elucidation of the mechanisms of immune evasion may lead to a better understanding of the pathobiology of Lyme disease.

The *vls* (Vmp-like sequence) locus of *B. burgdorferi* B31 is on the linear plasmid lp28-1, a plasmid associated with infectivity in the mouse model (26, 27, 42, 52). The *vls* locus consists of an expression site (*vlsE*) and 15 unexpressed (silent) *vls* cassettes. The silent cassettes have high homology to the central cassette region of *vlsE*. Within the cassettes, there are six variable regions interspersed between highly conserved regions (52). During experimental infection of mice, antigenic variation occurs in the *vlsE* gene through a series of gene conversion events between segments of the silent cassettes and the expression site. The resulting recombination leads to changes in the sequence of the expression cassette but no alterations in the sequences of the silent cassettes (53). *vlsE* variation has been shown to occur within 4 days of experimental infection of mice with *B. burgdorferi* B31 and continues throughout the course of infection but has not been observed in vitro or in the tick vector (21, 53, 54). The conservation of *vls* sequences in other strains and species of *Borrelia* indicate that the *vls* locus is important for the life cycle of Lyme disease agents (23, 25, 49).

Lyme disease patients mount a robust antibody response directed towards VlsE (29, 33), and patient sera have been shown to react strongly with the IR6 invariable region of the protein (1, 16, 28, 31, 32, 39, 40, 44). With experimentally infected mice, Triton X-114 extraction studies indicate that VlsE is present at high levels in joint and ear tissues but not in heart tissue (9), suggesting differential expression. Cross-absorption studies by McDowell et al. (34) have shown that antibodies specific for the variable regions of VlsE are generated during the course of experimental infection in mice. The three-dimensional structure of VlsE reveals the localization of the variable regions in the membrane-distal portion of the protein, covering a large portion of the invariable regions (13). The ability of *B. burgdorferi* to survive in the presence of an active anti-VlsE antibody response indicates that *vls* antigenic variation may lead to changes in surface-exposed epitopes of VlsE that protect the protein from recognition by anti-VlsE antibodies. The persistent infection seen in Lyme disease patients may be, in part, a result of *vls* antigenic variation.

While *vls* antigenic variation has been hypothesized as an immune evasion mechanism, the importance of the *vls* locus as a virulence factor during mammalian infection has not been

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clearly defined. *B. burgdorferi* B31 clones with a full complement of plasmids can be cultured from every tissue site examined in immunocompetent C3H/HeN mice months to years after inoculation; however, the absence of lp28-1 (lp28-1$^-$) in B31 clones in immunocompetent mice correlates with an intermediate infectivity phenotype in which *Borrelia* can be cultured from the joints, but rarely from other sites, 2 weeks after infection (26, 27, 42). Interestingly, the lp28-1$^-$ clone 5A8 could be cultured from all examined tissue sites of C3H severe combined immunodeficiency mice and also grew normally in dialysis membrane chambers implanted into rats (where the organisms would not be exposed to antibodies or immune cells) (41). Taken together, these results indicate that lp28-1 is required for full infectivity in the presence of an effective immune response, implicating its involvement in immune evasion; however, whether the loss of the *vls* locus or the loss of another lp28-1 gene(s) is responsible for this decreased virulence has not been determined.

Transformation of low passage, infectious isolates of *B. burgdorferi* occurs at low frequencies, limiting the ability to perform genetic studies of factors affecting infectivity (4, 12, 19, 30, 48). Recently, Grimm et al. (15) determined that disruption of *ospC* in an infectious *B. burgdorferi* B31 clone resulted in loss of the ability of the clone to infect mice, whereas complementation with *ospC* restored infectivity. In similar studies, Pal et al. (38) found that *ospC* mutation affected the ability of *B. burgdorferi* 297 to migrate from the tick midgut to the salivary glands during feeding, but the effect on infection of mice was not reported. Yang et al. (51) showed that inactivation of the *ospAB* operon had no apparent effect on the course of infection of mice, but it greatly decreased midgut colonization in ticks. These recent studies indicate that it is feasible to disrupt and complement *B. burgdorferi* genes in infectious isolates; however, the frequency at which these mutations were obtained was not indicated.

It has been shown that the presence of plasmids lp25 and lp56 are barriers to the transformation of low passage *B. burgdorferi* B31 with the shuttle plasmid pBSV2 (30). In addition to the role of lp25 as a transformation barrier, this plasmid is also required for infection in the mouse model (26, 42). Purser et al. (41) have shown that complementation of the noninfectious clone B31-5A13, which lacks lp25, with a DNA segment containing the *pncA* homolog BBE22 is able to restore infectivity in mice, representing the first restoration of virulence by gene complementation in Lyme disease *Borrelia* (41).

Shuttle vectors containing BBE22 provide a useful tool for the identification and characterization of *B. burgdorferi* virulence factors. *B. burgdorferi* strains lacking lp25 and lp56 can be readily transformed with the shuttle vector pBSV2 (30), and the inclusion of *pncA* in pBSV2 overcomes the low-infectivity phenotype associated with the absence of lp25 (41). Here, we report the use of a *pncA* shuttle vector to determine the ability of *vlsE* to restore infectivity to a *B. burgdorferi* clone lacking lp28-1. In addition, the production of a merodiploid clone containing both lp28-1 and a *trans* copy of *vlsE* on a shuttle plasmid was utilized to examine the importance of the *cis* versus the *trans* location of *vlsE* relative to the *vls* silent cassettes in *vls* gene conversion events.

TABLE 1. Bacterial strains used in this study

B. burgdorferi B31 clone	Missing plasmid(s)
5A4	None
5A8	lp28-1
5A10	lp25, lp56
5A10/pBBE22:vlsE-2	lp25, lp56
5A10/pBBE22:vlsE-5	lp25, lp56, lp28-1
5A13	lp25
5A13/pBSV2	lp25, lp56, lp28-1
5A13/pBBE22	lp25, lp56

MATERIALS AND METHODS

Bacterial strains. *Borrelia burgdorferi* B31 was isolated initially from *Ixodes scapularis* and was cultured in Barbour-Stoenner-Kelly (BSK) II medium as described previously (37). The infectivities and plasmid profiles of the *B. burgdorferi* B31 low-passage clones used in this study were determined previously (42) (Table 1). Clone 5A4 contains all of the plasmids examined and has a high-infectivity phenotype; 5A8 (lp28-1$^-$) is an intermediate-infectivity clone, whereas 5A10 (lp25$^-$ and lp56$^-$) and 5A13 (lp25$^-$) are low-infectivity clones. Due to the lack of lp25 and lp56, 5A10 is highly transformable while 5A13, which is missing only lp25, exhibits an intermediate transformation phenotype (30). Clone 5A13 was transformed with the shuttle vector pBSV2 (46) or pBBE22, consisting of pBVS2 and a 2.1-kb *B. burgdorferi* DNA insert containing BBE22, as described previously (41). *Escherichia coli* XL1Blue (Stratagene, La Jolla, Calif.) and *E. coli* One Shot TOP10 F′ (Invitrogen, Carlsbad, Calif.) were utilized to maintain shuttle vector constructs. *E. coli* One Shot TOP10 (Invitrogen) was used for the recovery of the shuttle vector constructs from *B. burgdorferi* transformants for *vlsE* sequencing.

Plasmid construction. The shuttle plasmid pBBE22:vlsE was constructed from the *Borrelia* shuttle vector pBSV2 (46) by the addition of *vlsE* and a 2.1-kb section of lp25 that contains BBE22. The *vlsE* gene was amplified by PCR from *B. burgdorferi* B31 5A3 with the forward primer 4710 (5′-GGG GTA CCA GAA ATG AAA AAT TCT CTG CAC CTA C AC TT-3′) and the reverse primer 4713 (5′-GCT CTA GAG AGG GCA TAG TCG TGT CCA TAC A-3′). These primers contain KpnI and XbaI restriction sites (underlined sequences near the respective 5′ ends of 4710 and 4713) and amplify the *vlsE* gene from 93 bp upstream of the start codon and 33 bp downstream of the stop codon of the 1,068-bp open reading frame of the allele *vlsE1* (GenBank accession number U76405). This region includes the native promoter and leader sequence (20, 52). pBSV2 and the *vlsE* PCR product were digested with KpnI and XbaI for 3 h at 37°C. pBSV2 was treated with shrimp alkaline phosphatase (Promega, Madison, Wis.), and the treated vector and insert were ligated by using a Rapid DNA ligation kit (Roche, Nutley, N.J.). The resulting plasmid, pMBL20, was transformed into *E. coli* XL1Blue, and the sequence of the *vlsE* insert was confirmed. The *B. burgdorferi* DNA fragment containing BBE22 was amplified with primers 4735 and 4734 as described previously (41), digested with KpnI, ligated into the KpnI site of pMBL20, and transformed into *E. coli* TOP10 F′. The presence of BBE22 was confirmed by PCR. Plasmid DNA for use in *B. burgdorferi* transformations was prepared from cultures of *E. coli* TOP10 F′ transformed with pBBE22:vlsE by using a Wizard Plus MaxiPrep DNA purification system (Promega) and was followed by ethanol precipitation of the plasmid DNA and resuspension in 30 μl of H_2O. Plasmid DNA was stored at −80°C until *B. burgdorferi* transformation.

***B. burgdorferi* transformation.** Electroporation of *B. burgdorferi* was performed as described previously (30). Following electroporation, bacterial cells were immediately resuspended in 1 ml of prewarmed (34°C) BSK II liquid medium and incubated for 48 h at 34°C. The cultures were plated in a soft agar overlay (10, 37) on BSK II plates with and without kanamycin (0.2 mg/ml) and incubated at 34°C for 2 to 4 weeks. Well-isolated colonies were selected by using sterile pipette tips and were cultured for 5 days in BSK II liquid medium with 0.2 mg of kanamycin/ml prior to storage in BSK II-10% glycerol. Plasmid DNA was isolated by using a Wizard Plus MiniPrep DNA purification system (Promega), and the presence of pBBE22:vlsE was determined by PCR amplification of the kanamycin cassette with primers 4795 and 4796 as described previously (30).

Western blot analysis. Protein samples were separated by electrophoresis using sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis at a constant voltage of 180 mV. The immunoblots were processed according to the method of Norris et al. (36) with mouse anti-VlsE1-His serum (1:10,000 dilution) as the primary antibody. Detection of reactivity was analyzed by using ECL Western blotting detection reagents (Amersham International, Princeton, N.J.)

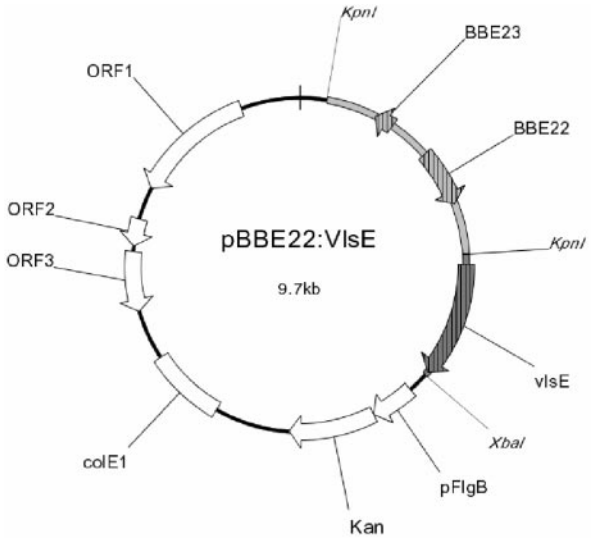

FIG. 1. pBBE22:vlsE plasmid map. KpnI and XbaI restriction sites used during cloning of segments containing BBE22 and *vlsE* into the pBSV2 shuttle vector are shown (20, 41, 46).

as described by the manufacturer. Surface proteinase K digestion of intact *B. burgdorferi* was carried out as described previously (36).

Mouse infections. *Borrelia* strains were tested for infectivity with C3H/HeN (wild-type) and C3H/Smn.ClcrHsd-*scid* (SCID) mice (Harlan, Indianapolis, Ind.) by intradermal needle inoculation (10^5 organisms/mouse) and culture of joint, heart, ear, and bladder tissues 2 or 8 weeks postinoculation, as previously described (37). Tissues from mice inoculated with *B. burgdorferi* transformed with pBSV2 or its derivatives were cultured in the presence of kanamycin (0.2 mg/ml) for 2 weeks. The presence or absence of viable spirochetes was determined by dark-field microscopy. Serum samples were obtained by tail vein bleeding at 2, 4, and 8 weeks after inoculation.

***vlsE* sequence determination.** To determine if sequence variation occurred in the *vlsE* gene on pBBE22:vlsE, total plasmid DNA from *B. burgdorferi* transformed with this plasmid was isolated with the Wizard Plus MiniPrep DNA purification system and transformed into *E. coli* One Shot Top10 as described by the supplier. Positive transformants containing pBBE22:vlsE were selected by growth on Luria-Bertani agar plates with 40 μg of kanamycin/ml. Plasmid DNA from individual clones was isolated by using a Wizard Plus MiniPrep DNA purification system, and the *vlsE* cassette was sequenced with primers 4120 and 4066 (52).

To determine if *vlsE* antigenic variation occurred in *B. burgdorferi* 5A13/pBBE22, individual clones were isolated on BSK II soft agar plates and the *vlsE* gene from lp28-1 was amplified with the primers 4910 (5′-GCT CTA GAG CGA AAT GAA AAA TTC TCT GCA CCT ACA CTT-3′) and 4341 (5′-CGG AAG CTT CAA TCA TGA GGG CAT AGT CGT GTC CAT ACA-3′). The cassette regions of these PCR products were then sequenced with primers 4120 and 4066 (52).

Antibody detection by ELISA. Anti-VlsE and anti-*Borrelia* antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described by Lawrenz et al. (29). Briefly, ELISA plates were coated with either 50 ng of VlsE1-His recombinant protein or with 500 ng of lysed *B. burgdorferi* B31 5A4 and blocked overnight with 1% milk, and 100 μl of a 1:800 dilution of serum was added to each well. Washing and detection were performed as described previously (29).

Quantitation of *Borrelia*. DNA was purified from ankle tissues as described previously (35) with minor modifications. Briefly, individual tissues were incubated in 0.1% collagenase A at 37°C overnight followed by the addition of an equal volume of a 0.2-mg/ml solution of proteinase K. After an overnight incubation at 55°C, DNA was recovered by phenol-chloroform extraction and ethanol precipitation. After digestion with DNase-free RNase (Sigma Chemical, St. Louis, Mo.) at 1 mg/ml, samples were again extracted and DNA was recovered by precipitation. This precipitate was resuspended in 0.5 ml of water, and the DNA content was determined by measuring the absorbance at 260 nm.

Quantitative DNA analyses were performed by using the LightCycler PCR system (Roche Diagnostics, Indianapolis, Ind.) as previously described (50). Briefly, amplification was performed with 100 ng of sample DNA in a 10-μl final volume containing 50 mM Tris (pH 8.3), 3 mM $MgCl_2$, 4.5 μg of bovine serum albumin, a 200 μM concentration of each deoxynucleoside triphosphate, a 1:20,000 dilution of SYBR Green I (Molecular Probes, Eugene, Oreg.), a 5 μM concentration of each primer, and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen). Amplification was performed for 40 cycles, with each cycle consisting of heating at 20°C per s to 95°C with a 1-s hold, cooling at 20°C per s to 60°C with a 4-s hold, and heating at 1°C per s to 82°C. To minimize the inclusion of nonspecific products, the fluorescent signal was collected at 82°C at the end of each cycle. Melting curves were used to confirm the specificities of the assessed PCR products. The relative starting copy number present in each sample was determined by cycle threshold detection by using LightCycler analysis software. Copy numbers for the single-copy mouse *nidogen* gene and *B. burgdorferi recA* were calculated by using LightCycler relative quantification software (Roche) and known external standards. The reported *recA* values were corrected by normalization to the *nidogen* gene copy number. The oligonucleotide primers used to detect murine *nidogen* were nidoF (5′-CCA GCC ACA GAA TCA CAT CC-3′) and nidoR (5′-GGA CAT ACT CTG CTG CCA TC-3′). The oligonucleotide primers used to detect *B. burgdorferi recA* were nTM17.F (5′-GTG GAT CTA TTG TAT TAG ATG AGG CTC TCG-3′) and nTM17.R (5′-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3′) (35).

RESULTS

Complementation with *vlsE*. Previous studies had shown that a DNA segment containing the lp25 gene BBE22 was able to restore infectivity in $lp25^-$ clones of *B. burgdorferi* B31 (41) and that the absence of lp28-1 (containing the *vls* locus) correlated with an intermediate infectivity phenotype (26, 27, 42). Furthermore, clones containing lp25 are refractory to transformation (30). To determine the role of *vlsE* during mammalian infection, we developed a strategy in which shuttle plasmids containing BBE22 either with or without *vlsE* were transformed into *B. burgdorferi* lacking lp25. The *B. burgdorferi* clones employed in this study are listed in Table 1.

The shuttle plasmid pBSV2 (46) was modified to contain *vlsE* and BBE22 (Fig. 1) and transformed *B. burgdorferi* B31 5A10, which is missing lp25 and lp56 (42). The plasmid profiles of individual pBBE22:vlsE transformants were determined by PCR (42), and two isolates were chosen for further analysis. 5A10/pBBE22:vlsE clone 2 (5A10/pBBE22:vlsE-2) is missing only the plasmids lp25 and lp56 and thus contains two copies of *vlsE*, one on lp28-1 and one on the shuttle plasmid. A second clone, 5A10/pBBE22:vlsE clone 5 (5A10/pBBE22:vlsE-5), had spontaneously lost lp28-1; therefore, it is missing the plasmids lp25, lp56, and lp28-1 and contains a single copy of the *vlsE* gene on the shuttle plasmid and no *vls* silent cassettes. The expression of VlsE in each of these clones was determined by Western blot analysis by using VlsE-monospecific serum (Fig. 2). The presence of VlsE in 5A10/pBBE22:vlsE-5 demon-

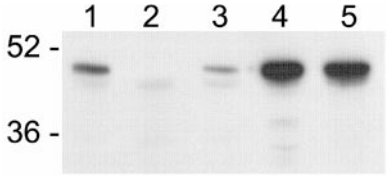

FIG. 2. Western blot analysis demonstrating VlsE expression in *vlsE*-complemented *B. burgdorferi* clones. Lane 1, 5A4 (wild type, containing all of the native *B. burgdorferi* plasmids); lane 2, 5A8 (lacking the VlsE-encoding plasmid lp28-1); lane 3, 5A13/pBBE22 ($lp25^-$, complemented with BBE22); lane 4, 5A10/pBBE22:vlsE-2 ($lp25^-$ and $lp28\text{-}1^+$, complemented with BBE22 and *vlsE*); lane 5, 5A10/pBBE22:vlsE-5 ($lp25^-$ and $lp28\text{-}1^-$, complemented with BBE22 and *vlsE*). Numbers at the left are molecular weight standards (in thousands). A quantity of bacterial lysate equivalent to 10^7 organisms was loaded in each lane.

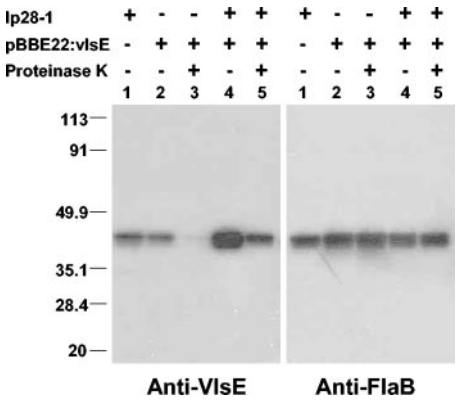

FIG. 3. Effect of surface proteolysis on VlsE in *vlsE*-complemented *B. burgdorferi* clones. Intact *B. burgdorferi* were treated with proteinase K for 40 min and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10^7/lane) and Western blotting. Two identical blots were reacted with either mouse polyclonal anti-VlsE (left panel) or a mouse monoclonal antibody against the flagellar protein FlaB (right panel). Lanes 1, clone 5A4 (containing all native *B. burgdorferi* plasmids); lanes 2 and 3, clone 5A10/pBBE22:vlsE-5 (lacking lp25, lp56, and lp28-1 but containing shuttle vector pBBE22:vlsE); lanes 4 and 5, clone 5A10/pBBE22:vlsE-2 (lacking lp25 and lp56 but containing both lp28-1 and pBBE22:vlsE). Proteinase K treatment of intact *B. burgdorferi* (lanes 3 and 5) dramatically reduced VlsE immunostaining but did not affect levels of the periplasmic protein FlaB. Numbers on the left are molecular weight markers (in thousands).

strates that the promoter region of *vlsE* present in the construct is sufficient for the expression of VlsE in *B. burgdorferi* in vitro. Higher expression of VlsE was observed in 5A10/pBBE22:vlsE-2 (containing lp28-1 [lp28-1^+]) than in 5A10/pBBE22:vlsE-5 (lp28-1^+), indicating that the expression from the native and *trans* copies of VlsE is additive.

Although the copy of *vlsE* on pBBE22:vlsE had both the native promoter and the native leader sequence, it is possible that *trans* expression of VlsE could affect its surface localization. Previous studies had demonstrated that VlsE is degraded when intact *B. burgdorferi* are exposed to proteinase K, whereas FlaB, a periplasmic protein, is not affected by this treatment (52). As shown in Fig. 3, VlsE expressed solely from pBBE22:vlsE (in clone 5A10/pBBE:vlsE-5) was readily degraded when intact bacteria were treated with proteinase K, consistent with its surface accessibility. When both the native (lp28-1) and *trans* copies of *vlsE* were present, some of the larger quantity of VlsE expressed still remained after proteinase K treatment. FlaB was consistently present in similar quantities with or without proteinase K, indicating that the protease treatment did not affect periplasmic proteins.

Effect of *vlsE* complementation on virulence in the mouse model. To determine if virulence could be restored in an lp28-1^- clone by complementing in *trans* with *vlsE*, immunodeficient C3H/Smn.ClcrHsd-*scid* mice (SCID) and C3H/HeN immunocompetent mice were inoculated with 5A10/pBBE22:vlsE-5 for 2 weeks and compared to infection by *B. burgdorferi* 5A13/pBSV2 (vector control) and 5A13/pBBE22 (BBE22 control). As expected, the positive control clone 5A4 (containing all native *B. burgdorferi* plasmids) was fully infectious in both mouse strains. The clone of 5A13/pBSV2, used as a negative control in this study, did not contain lp25 and was later found to have also lost lp28-1 spontaneously (Table 1). This clone was not recovered from SCID or C3H/HeN mice, whereas the BBE22 complemented isolate 5A13/pBBE22 (which had retained lp28-1) was recovered from all six mice in both groups (Table 2). Organisms were isolated from all tissues of the six SCID mice infected with 5A10/pBBE22:vlsE-5; this clone was reisolated from all C3H/HeN mice inoculated, but only 57% of the tissues were positive for *B. burgdorferi* infection by culture (Table 2). In contrast, the clone 5A10/pBBE22:vlsE-2, which contained both lp28-1 and the pBBE22:vlsE construct, was recovered from all sites in both SCID and C3H/HeN mice. These results provided evidence that *trans* complementation with *vlsE* does not increase the infectivity of a clone lacking lp28-1 to the level observed for an lp28-1-positive clone.

To examine the infectivities of BBE22- and *vlsE*-complemented clones over a longer time period, we infected C3H/HeN mice with the *B. burgdorferi* clones 5A4, 5A8, 5A13/pBSV2, 5A13/pBBE22, and 5A10/pBBE22:vlsE-5 for 8 weeks and cultured tissues to determine if these clones displayed differing infectivity phenotypes. The *B. burgdorferi* 5A4 wild-type control and 5A13/pBBE22 were isolated from all tissues of all mice, while we were unable to isolate any organisms from mice infected with the 5A13/pBSV2 vector-only control (Table 3). Clone 5A8, which is missing only lp28-1, also was not isolated from any tissues at this time interval. 5A10/pBBE22:vlsE-5 showed a decrease in infection rate at 8 weeks postinfection relative to 2 weeks postinfection and was isolated only from the joint of one mouse at 8 weeks postinfection (Table 3). These results indicate that *B. burgdorferi* lacking lp28-1 was effectively cleared from the tissues examined by 8 weeks postinfection in this model and that complementation with *vlsE* does not restore the ability of lp28-1^- clones to cause persistent infection comparable to wild-type levels in immunocompetent mice.

Lack of *vlsE* sequence variation on the shuttle plasmid. It has been shown previously that *vlsE* variants could be isolated

TABLE 2. Effect of plasmid content and *vlsE* complementation on *B. burgdorferi* infection at 2 weeks postinfection

B. burgdorferi B31 clone	Plasmid		Shuttle vector			No. of cultures positive/no. tested for sample site indicated from:									
						SCID mice					C3H/HeN mice				
	lp25	lp28-1	pBSV2	BBE22	*vlsE*	Heart	Bladder	Ear	Joint	Total	Heart	Bladder	Ear	Joint	Total
5A13/pBSV2	−	−	+	−	−	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
5A13/pBBE22	−	+	+	+	−	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/5[a]	6/6	6/6
5A10/pBBE22:vlsE-2	−	+	+	+	+	6/6	6/6	6/6	6/6	6/6	6/6	6/6	3/6	6/6	6/6
5A10/pBBE22:vlsE-5	−	−	+	+	+	6/6	6/6	6/6	6/6	6/6	3/7	5/7	2/7	6/7	7/7

[a] One culture was contaminated and is thus not included.

TABLE 3. Effect of plasmid content and *vlsE* complementation on *B. burgdorferi* infection of C3H/HeN mice at 8 weeks postinfection

B. burgdorferi B31 clone	Plasmid		Shuttle vector			No. of 8-wk cultures positive/no. tested for sample site indicated				
	lp25	lp28-1	pBSV2	BBE22	*vlsE*	Heart	Bladder	Ear	Joint	Total
5A4	+	+	−	−	−	6/6	6/6	6/6	6/6	6/6
5A8	+	−	−	−	−	0/6	0/6	0/6	0/6	0/6
5A13/pBSV2	−	−	+	−	−	0/6	0/6	0/6	0/6	0/6
5A13/pBBE22	−	+	+	+	−	6/6	6/6	6/6	6/6	6/6
5A10/pBBE22:vlsE-2	−	+	+	+	+	6/6	6/6	6/6	6/6	6/6
5A10/pBBE22:vlsE-5	−	−	+	+	+	0/6	0/6	0/6	1/6	1/6

from mice within 4 days postinfection and that all clones exhibited *vlsE* sequence variation by 2 weeks postinfection (54). Since 5A10/pBBE22:vlsE-2 retained lp28-1 and the *vls* silent cassettes, we determined if sequence variation occurs on the *vlsE* gene of pBBE22:vlsE in this clone following mouse infection. Plasmid DNA from *B. burgdorferi* clones reisolated from C3H/HeN mice 2 and 8 weeks postinfection was used to transform *E. coli*. For *vlsE* sequence analysis, 10 individual clones (four from the ear, three from the bladder, and three from the joint) were selected at 2 weeks postinfection, and 16 individual clones (five from the heart, five from the ear, five from the joint, and one from the bladder) were selected at 8 weeks postinfection. pBBE22:vlsE was consistently recovered from all *B. burgdorferi* clones examined, regardless of whether kanamycin selection was utilized during reisolation or cloning of *B. burgdorferi*. All 26 *vlsE* sequences were identical to the sequence of the *vlsE* originally cloned into pBBE22:vlsE (data not shown), indicating that sequence variation did not occur in the *trans* copy of *vlsE* during infection.

***vlsE* sequence variation in lp28-1 in *B. burgdorferi* lacking lp25 and lp56.** The lack of sequence variation in the shuttle plasmid-encoded *vlsE* may be a result of the absence of lp25 and lp56 in clone 5A10. To resolve the question of whether the loss of these plasmids is responsible for the lack of *vlsE* sequence variation on pBBE22:vlsE, we determined the sequence of the native *vlsE* gene on lp28-1 from clones lacking lp25 and lp56 that have been recovered from mice. A direct analysis of the *vlsE* sequence on lp28-1 in 5A10/pBBE22:vlsE-2 was complicated by the inability to design primers that would distinguish between the *cis* and *trans* copies of *vlsE* in the complemented clone. Therefore, we analyzed the sequence of *vlsE* from 5A13/pBBE22 clones (which lacked the *trans* copy of *vlsE*) reisolated from mice. The *vlsE* gene was amplified by PCR from three clones isolated from mice 2 weeks postinfection and three clones isolated from mice 8 weeks postinfection. The *vlsE* cassette regions were sequenced and compared to the *vlsE* sequence from the original 5A13/pBBE22 isolate. Sequence variation was observed in all six of the isolated clones (Fig. 4), with a higher number of sequence differences being observed in the clones isolated 8 weeks postinfection. These results indicate that (i) lp25 and lp56 are not required for *vlsE* variation, and (ii) the lack of the *vlsE* variation on the shuttle plasmid is not a result of the absence of these plasmids in the complemented clone.

Antibody response to *vlsE*-complemented clones. To determine whether C3H/HeN mice infected with 5A10/pBBE22:vlsE-2 or 5A10/pBBE22:vlsE-5 elicited an anti-VlsE immune response, the presence of anti-VlsE antibodies was determined by ELISA (Fig. 5). As expected, mice infected with the wild-type clone 5A4 exhibited strong anti-*B. burgdorferi* and anti-VlsE antibody responses that increased with time. Mice infected with the clone 5A13/pBSV2 (lacking lp25 and lp28-1) developed a weak anti-*B. burgdorferi* response that increased slightly over time but did not exhibit a response against VlsE. The anti-*B. burgdorferi* response observed may have been elicited by the high inoculum used (10^5/mouse). 5A13/pBBE22

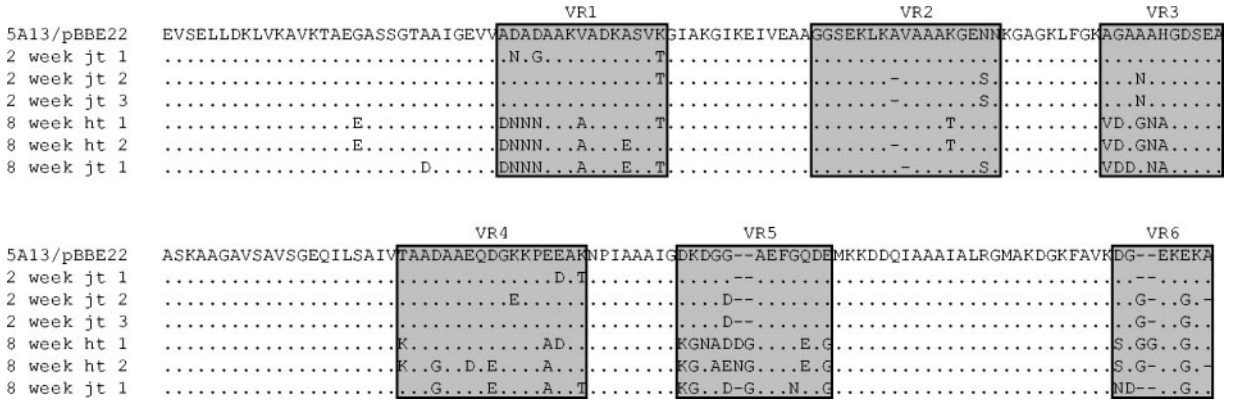

FIG. 4. Alignment of predicted amino acid sequences of cassette regions of VlsE from *B. burgdorferi* 5A13/pBBE22 reisolated from joints (jt) and hearts (ht) of mice 2 and 8 weeks postinoculation. The cassette region of VlsE from *B. burgdorferi* 5A13/pBBE22 was used to infect mice. Identical amino acid sequences are indicated by periods, and gaps are indicated by hyphens. The variable regions (52) are indicated by shaded boxes.

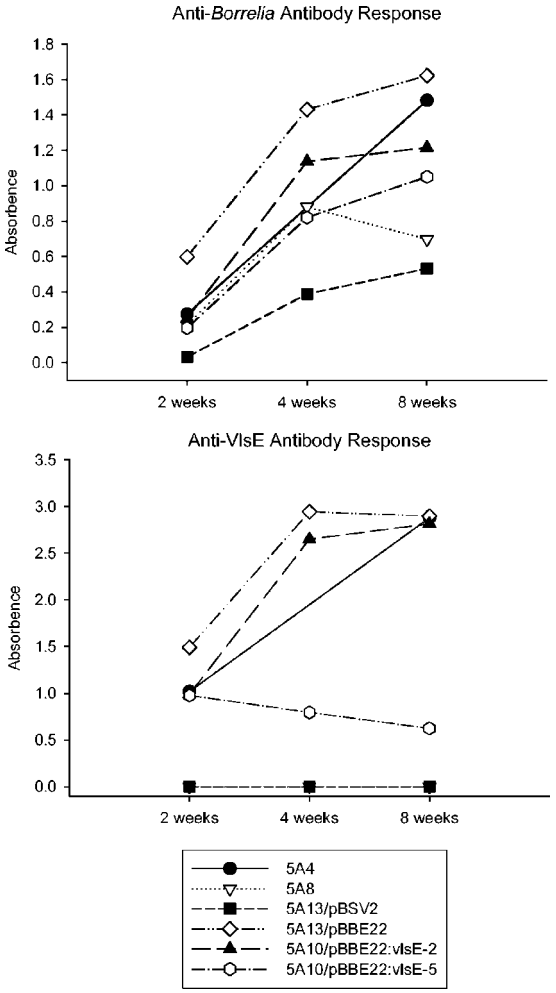

FIG. 5. IgG response elicited by infected mice to *B. burgdorferi* and VlsE recombinant protein. Graphs represent mean absorbencies obtained by ELISAs using *B. burgdorferi* sonicates and recombinant VlsE as antigens (see Materials and Methods).

infection resulted in a strong IgG antibody response to both *B. burgdorferi* and VlsE. Mice infected with 5A10/pBBE22:vlsE-5 (lacking lp28-1 but having a *trans* copy of *vlsE*), which was unable to persist in the mouse model for 8 weeks, developed an early immune response to VlsE that decreased over time. Similar to results with 5A13/pBBE22, mice infected with 5A10/pBBE22:vlsE-2 developed a strong IgG response against both *B. burgdorferi* and VlsE over the 8-week course of infection. These results, together with the lack of sequence variation in the *trans* copy of *vlsE* in 5A10/pE22:vlsE-2, indicate that *B. burgdorferi* is able to persist in the mammalian host when a nonvariant form of VlsE is expressed for extended periods of time.

Quantitation of *Borrelia* in infected mice. The reisolation of spirochetes by culturing tissues is a qualitative measure and does not define the actual numbers of *Borrelia* present in each tissue. More subtle differences in infectivity levels could be detected by using a more sensitive quantitative assay. We therefore utilized quantitative PCR to determine the numbers of spirochetes in the tibiotarsal joints of infected mice (Fig. 6). At 2 weeks postinfection, decreased numbers of 5A10/pBBE22:vlsE-2 were observed compared to 5A4 and 5A13/pBBE22 (BBE22 control); however, 5A10/pBBe22:vlsE-2 numbers increased to comparable levels by week 4. By 8 weeks postinfection, 5A10/pBBE22:vlsE-2 numbers did decrease but a similar decrease was also observed for 5A13/pBBE22. The presence of lower numbers of 5A10/pBBE22:vlsE-2 at 2 weeks postinfection may suggest a defect either in the translocation to the joints (lower number of spirochetes disseminating to the tissue) or in multiplication within the joints of infected animals; however, the apparent expression of a static form of VlsE in the merodiploid clone did not prevent the persistence of 5A10/pBBE22:vlsE-2 within these tissues after colonization.

DISCUSSION

B. burgdorferi clones lacking lp28-1 have a decreased ability to infect C3H/HeN mice but not immunodeficient SCID mice, implicating an interaction between an lp28-1-encoded virulence factor and the mammalian adaptive immune system (26, 42). The *vls* locus on lp28-1 shares homology to the *vlp-vsp* immune evasion system of relapsing fever spirochetes and is a strong candidate to mediate these interactions (34, 47, 52–54); however, attempts to determine the role of the *vls* locus during *B. burgdorferi* infection have been limited because of the low transformation efficiency of infectious clones (4, 12, 19, 30, 48). This transformation barrier has been attributed to the plasmids lp25 and lp56 and is likely due to putative restriction-modification systems encoded on these plasmids (30). Transformation efficiency in clones that lack these two plasmids is dramatically increased, but the organisms are noninfectious due to the loss of lp25. However, recent work that identified the lp25-encoded virulence determinant(s) has indirectly provided a system that allows us to efficiently perform genetic manipula-

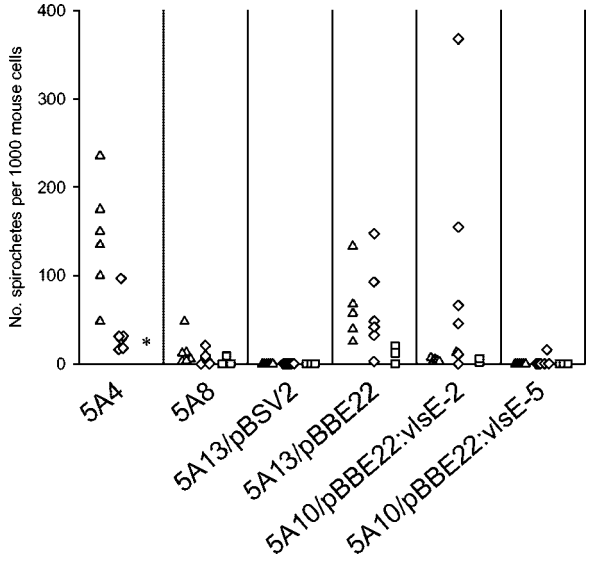

FIG. 6. Quantitation of *B. burgdorferi* in tibiotarsal joints of C3H/HeN mice. All tissues were assessed in a blinded fashion, and each symbol represents the average value for a single animal. Triangles, samples taken 2 weeks postinfection; diamonds, samples taken 4 weeks postinfection; squares, samples taken 8 weeks postinfection; *, absence of samples for this strain and time point.

tions in clones that lack lp25 and lp56 and are still infectious. Purser et al. (41) demonstrated that complementing clones lacking lp25 with the BBE22 and BBE23 open reading frames on the pBSV2 shuttle vector (pBBE22) is able to restore virulence in clones lacking lp25. By modifying pBBE22 to include *vlsE* (Fig. 1), we were able to efficiently complement infectious *B. burgdorferi* clones with *vlsE* and assess the effect on mammalian infection.

Complementation of a *B. burgdorferi* clone lacking lp28-1 with *vlsE* was not sufficient to restore full infectivity in mice (Tables 2 and 3). *vlsE*-complemented spirochetes were isolated from the heart and bladder 2 weeks postinfection at higher frequencies than previously reported for lp28-1-lacking clones (26, 27, 41, 42), suggesting that *vlsE* could restore lp28-1-associated virulence (Table 2). However, unlike the clones containing lp28-1, the lp28-1$^-$-complemented clones were unable to persist in immunocompetent mice for 8 weeks postinfection (Table 3). Clearance of the complemented clones lacking lp28-1 corresponded to the development of an anti-VlsE immune response and suggests that the inability of the complemented spirochetes to undergo VlsE antigenic variation may be responsible for the decreased persistence of the complemented clones compared to wild-type organisms. Another possibility is that lp28-1 genes other than the *vls* locus may be required for persistent infection. Increased isolation of 5A10/pBBE22:vlsE-5 from tissues during early infection may also suggest that *vlsE* has functions in addition to immune evasion, such as trafficking to or multiplication in specific tissues. Crother et al. (9) showed that VlsE is prominently expressed by *B. burgdorferi* in skin or joint tissues but not in the heart; differential expression of *vlsE* may thus be related to tissue-specific pathogenesis. However, previous reports monitoring dissemination of clones lacking lp28-1 during early infection and with immunodeficient mice have demonstrated that lp28-1$^-$ clones are able to successfully disseminate to each of the tissues examined (26, 27, 42). Future studies of either *vlsE* inactivation on lp28-1 or complementation with the entire *vls* locus would allow us to determine whether other virulence factors are present on lp28-1 and if VlsE has additional virulence functions; however, attempts at either strategy have been unsuccessful to date (unpublished data).

Complementation of clones that retained lp28-1 with *vlsE* provided additional insight into *vlsE* variation in the mouse model. We have shown that *vlsE* recombination occurs in the absence of lp25 and lp56 (Fig. 4); however, variation was not detected with the *trans* copy of *vlsE*. The apparent necessity for the *cis* localization of the expression site on the same replicon as the *vls* silent cassette locus differs from the *vlp-vsp* antigenic variation systems of relapsing fever spirochetes, in which the silent genes are located on a different plasmid than the expression locus (2, 3, 43). In addition, it has been shown that *pilE* recombination can occur in *trans* in *Neisseria gonorrhoeae* via a circular intermediate (18), indicating that a novel recombination mechanism may be utilized by the *vls* system.

It is possible that the *vlsE* recombination process is dependent upon a stem loop structure (consisting of 37-bp inverted repeats and a 6-bp loop) found in the region between *vlsE* and the silent cassettes, as identified by Hudson et al. (20). The inverted repeat overlaps with a portion of the promoter sequence, as verified by primer extension analysis. Hudson et al. (20) also noted that *vlsE* expression was increased by the incubation of *B. burgdorferi* with human umbilical vein endothelial cells or cell membranes derived from these cells. As mentioned previously, Crother et al. (9) determined that VlsE is expressed in joint tissue at high levels during mouse infection and that *vlsE* transcript levels are also increased. The construct used in our study contains only 93 bp upstream of the *vlsE* reading frame and thus includes just one side of the stem loop structure; this characteristic may affect transcriptional regulation or sequence variation. In the immunoglobulin gene loci, V(D)J recombination is increased by DNA demethylation and germ line gene segment transcription (22, 24). *vlsE* recombination also appears to be regulated, because it is not detectable during in vitro culture but occurs at high rates during mouse infection (54). Therefore, one could speculate that *vlsE* recombination is also linked to transcription rates or DNA modification. In addition, the stem loop structure may be involved in the positioning of the *vlsE* cassette region and a donor silent cassette for gene conversion. Finally, the circular nature of the shuttle vector may have inhibited recombination between the shuttle plasmid and lp28-1. Future analysis on the *vlsE* recombination system may warrant the inclusion of linear *vlsE* shuttle plasmid constructs to definitively determine if the *vls* variation system requires a *cis* organization of the locus.

The inability of the *vlsE* gene on the shuttle plasmid to undergo sequence variation should have resulted in the expression of a static form of VlsE on the surface of the spirochete; indeed, VlsE expressed from the shuttle vector by in vitro-cultured *B. burgdorferi* was surface exposed, as indicated by surface proteolysis studies (Fig. 3). However, we did not observe clearance of the *vlsE* merodiploid clone from the mouse model. While these data may imply that antigenic variation is not required for persistence, additional factors could influence the results. First, while the expression of VlsE from the native gene appears to increase during infection (see previous paragraph), the in vivo expression levels from the shuttle plasmid are not known. The merodiploid clone may have been able to persist because the relative amount of *vlsE* expressed in vivo from the shuttle plasmid was significantly less than the amount expressed from the native gene. Furthermore, the coexpression of surface proteins, such as P66 and OspA, has been shown previously to mask epitopes from antibodies on the surface of *B. burgdorferi* (5), and similar interactions between VlsE from the shuttle plasmid with VlsE from the native gene (undergoing variation) and/or with other surface proteins may aid in masking epitopes on the static VlsE, resulting in evasion or slower clearance. Alternatively, selective pressure against VlsE during the progression of infection may promote the outgrowth of clones that have mutations in the shuttle plasmid that lead to decreased expression of *vlsE*; this possibility has not yet been examined. Quantitation of in vivo expression from the shuttle plasmid during infection may help to elucidate whether these admittedly speculative factors play a role in the in vivo survival of clones expressing an invariant form of VlsE.

In conclusion, the work reported here describes the use of BBE22-containing shuttle plasmids as one approach for assessing the role of other *B. burgdorferi* virulence determinants during infection. The inclusion of a gene of interest on the pBBE22 plasmid and the complementation of *Borrelia* lacking certain native plasmids were utilized in this study. However, it

is also possible to perform random or site-directed mutagenesis in a high-transformation-efficiency strain lacking lp25 and lp56, followed by complementation with pBBE22 to determine the ability of individual mutants to cause experimental infection. These types of analysis provide powerful tools for the identification and characterization of novel virulence factors in *B. burgdorferi* that have not been available in the past. Our results do not define fully the role of *vlsE* or *vls* antigenic variation in infectivity, but two important conclusions can be drawn from these data. First, complementation with *vlsE* does not fully restore infectivity of a clone lacking lp28-1 in immunocompetent mice, indicating that either *vlsE* variation or an additional gene on lp28-1 is important for virulence. Second, a *cis* requirement for the *vls* recombination mechanism has been indicated. As genetic techniques in *B. burgdorferi* develop further, we should be able to construct directed *vlsE* mutations that will aid in the further elucidation of the role of the *vls* system in virulence.

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